学　位　論　文

学位論文名

Immunogenic Tumor Cell Death Induced by Chemotherapy in Patients with Breast and Esophageal Cancer

（　乳癌および食道癌患者における化学療法が惹起する Immunogenic cell death の検討　）

福島県立医科大学大学院医学研究科

腫瘍専門医養成コース

青砥慶太

論　文　内　容　要　旨（和文）

学位論文題名	乳癌および食道癌患者における化学療法が惹起する Immunogenic cell death の検討

【背景】放射線治療やある種の抗癌剤は、直接的な腫瘍死に加えて、腫瘍特異的免疫系（特にT細胞系）を賦活化することで抗腫瘍効果を発揮する。この現象（いわゆるImmunogenic tumor cell death ; ICD）は、以前より知られていた。特に、局所照射により非照射野の遠隔転移が縮小する現象は、Abscopal効果として古くから知られていたが、頻度は稀で不明な点が多かった。しかし近年、担癌に伴う免疫抑制や免疫寛容の機序の解明が進むにつれ、抗癌剤投与後にもICDが誘導されていることが明らかになってきた。これは抗癌剤治療の基本原則である、患者が耐えられる最大量（maximum tolerated dose）を投与するという方法（免疫系が破綻する）ではなく、担癌生体の免疫応答を修飾・改善する至適量での抗癌剤治療という方法が、新たな選択肢として挙げられることを意味する。ICDの誘導には腫瘍局所のHMGB1やcalreticulinなどのMediatorの関与が重要視されているが、臨床的な検討はなされておらず、その意義は未知の部分が多い。
そこで、我々は術前化学療法(NAC)が実施された手術検体、および癌細胞培養系を対象としてICDの検討を行った。
【方法】①術前化学療法（NAC）を実施した乳癌と食道癌の術前生検標本と手術標本を抗HMGB1抗体と抗calreticulin抗体で免疫染色し、各々の発現強度を半定量化することで、比較検討した。また、臨床データとの関連を調べた。②3種類の代表的な乳癌細胞株（MDA-MB-231、MCF-7,SK-BR-3）を用いて抗癌剤(paclitaxel、doxorubicin)で治療し、培養上清中のHMGB1をELISAで、細胞表面のCalreticulin発現量をFlow cytometryで、癌細胞のApoptosisをAnnexin-V/7-AAD染色でFlow cytometryで定量化し、in vitroの実験系で抗癌剤によるICD関連の細胞反応を直接観察し検討した。
【結果】①HMGB1とCalreticulinいずれもNAC後に有意に発現強度が増強した。病理学的治療効果判定やOSとNAC前のHMGB1の染色強度とに統計的な有意差は認めなかった。②乳癌3株すべてにおいて50-80%のapoptosisが誘導される条件下であってもHMGB1の分泌量、Calreticulin発現量に細胞株および抗癌剤によって大きな差異が認められた。【結語】Chemo単独でも病理学的治療効果判定の程度にかかわらず、ICDが有意に誘導されていることが判明した。

（公表誌名、公表年月日、巻番号、ページ　　）

Introduction

Breast cancer and esophageal cancer is well known to be sensitive to chemotherapy and/or radiotherapy, and their combination with surgery was proven to have clinical benefits [1-6]. Multidiscipline procedure including chemotherapy, radiotherapy and surgery is regarded as a one of the standard of care for these patients. In clinical practice, it is generally accepted that there are responders and non-responders to chemotherapy with/without radiotherapy in these patients, and there is limited information describing the mechanisms and biomarkers to predict responders.

Although chemo-radiotherapy (CRT) is aimed at directly inducing apoptosis or necrosis, there is accumulating evidence to support the novel concept that CRT may induce immunogenic cell death (ICD) against tumor cells [7-18], where CRT could trigger uptake of antigenic components by dendritic cells (DC) and transfer antigenic signals to T-cell–mediated immunity, resulting in the expansion of antigen-specific CTLs and production of tumor-specific monoclonal antibodies. We and others have shown that HMGB1 and calreticulin induced by cytotoxic stresses such as CRT are important mediators to induce ICD [19]. However, there is limited information describing whether ICD could be induced by chemotherapy alone in clinical settings.

In the present study, we evaluated (i) whether expression of HMGB1 and calreticulin correlate to clinical outcomes in response to chemotherapy, and (ii) whether chemotherapy alone could upregulate HMGB1 and calreticulin in clinical and in vitro settings.

Materials and Methods

Patients and samples

The expression of HMGB1 and calreticulin was evaluated by immunohistochemistry in pre-treatment biopsy specimens and surgically resected specimens obtained from patients with breast cancer (n=52) or esophageal cancer (n=8) who treated with neoadjuvant chemotherapy between 2005 and 2015 at the Department of Organ Regulatory Surgery in Fukushima Medical University Hospital. Tumors were classified according to the TNM classification of malignant tumors (UICC 7th edition). Clinical and pathological information was retrospectively obtained by review of medical records, with the last follow-up in February 2016. Overall survival (OS) was defined as time from the date of surgery to the date of death. The median follow-up time was 51.0 months. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Fukushima Medical University.

Cell lines

Breast cancer cell lines, MDA-MB-231 (estrogen receptor (ER)-negative and HER2-negative), MCF-7 (ER-positive and HER2-negative) and SK-BR-3 (ER-negative and HER2-positive) were obtained as described previously [20] and cultured in RPMI 1640 medium with 10% fetal calf serum (FCS), 50 U/mL penicillin, and 50 μg/mL streptomycin.

Immunohistochemistry

Immunohistochemistry for HMGB1 and calreticulin was conducted using formalin-fixed paraffin-embedded (FFPE) specimens. Four-μm thick sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in methanol. Antigens were retrieved by autoclave for 5 min in 10 mM citrate buffer solution (105°C, pH 6.0). Sections were incubated with primary mouse

monoclonal anti-HMGB1 antibody (Clone 1D5, 1:500, Sigma-Aldrich) or with primary mouse monoclonal anti-calreticulin antibody (FMC 75, 1:10000, Abcam) at 4°C overnight, and subsequently detected by a horseradish peroxidase-coupled anti-mouse polymer (Envision, Dako, Heverlee, Belgium) followed by incubation with diaminobenzidine (Dako). All sections were counterstained with hematoxylin. The grade of HMGB1 and calreticulin expression was scored as 0 (0%–10% positive), 1+ (10%–30% positive), 2+ (30%–80% positive), or 3+ (> 80% positive) on tumor cells (Figure 1) in serial sections using 5 randomly selected areas at a magnification of 400x. Microscopic analyses were evaluated independently by two investigators (KA and KK) who had no prior knowledge of the clinical data.

In vitro treatments of breast cancer cell lines with chemotherapeutic agents

Three breast cancer cell lines, MDA-MB-231, MCF-7, and SK-BR-3 were incubated with RPMI medium in 6-well plates and treated with chemotherapeutic drugs, paclitaxel (0.1–1μM) or doxorubicin (0.1–1μM) in serum free medium (AIM-V®) on day 0. Dying cells were analyzed by Annexin-V (BD Pharmingen) and 7-aminoactinomycin D (7-AAD; BD Pharmingen) by flow cytometry, and the proportion of dying cells was determined by either Annexin-V positive or 7-AAD positive cells. Supernatants of treated breast cancer cell lines' cultures were measured for HMGB1 contents by ELISA (Shinotest) and cell surface expression of calreticulin was evaluated by flow cytometry with R-phycoerythrin (RPE)-conjugated anti-calreticulin mAb (Enzo life Sciences).

Statistical analysis

Paired t-test was used to determine differences of HMGB1 and calreticulin scores before and after NAC. Chi-square test was used for the evaluation

between chemo-response and HMGB1 expression, and between chemo-response and calreticulin expression. Unpaired t-test was used for HMGB1 and calreticulin expression between control and chemotherapy-treated cell lines. Cumulative survival was estimated by the Kaplan-Meier method, and differences between two groups were analyzed by a log-rank test. All statistical analyses were two-sided and were conducted using Graphpad Prism v6.0 (Graphpad Software Inc., La Jolla, CA, USA). P-values less than 0.05 were considered statistically significant.

Results

HMGB1 and calreticulin expression before and after chemotherapy

To evaluate HMGB1 and calreticulin expression within tumor microenvironments induced by chemotherapy alone, immunohistochemical analysis was conducted in pre-treatment biopsy specimens and surgically resected specimens obtained from patients with breast and esophageal cancer who treated with neoadjuvant chemotherapy. In order to semi-quantitatively evaluate HMGB1 and calreticulin expression, we have classified them into 4 grades (0, 1+, 2+ and 3+) as described in Material and Methods section and the representative immunostainings using anti-HMGB1 and anti-calreticulin mAbs are shown in Figure 1.

Of importance, both HMGB1 and calreticulin expression were significantly upregulated after chemotherapy compared to pre-treatment samples in breast and esophageal cancer as shown in several representative cases (Figure 2A and 2B). Summarized data from all samples showed that the degree of HMGB1 and calreticulin expression were significantly upregulated after chemotherapy compared to pre-treatment samples in breast and esophageal cancer (Figure 3). Thus, it is strongly suggested that chemotherapy alone could upregulate

HMGB1 and calreticulin expression in tumor microenvironments with breast cancer and esophageal cancer.

HMGB1 and calreticulin expression relating to pathological responses after chemotherapy and patient`s survivals

Since number of patients in breast cancer was enough to evaluate clinical data, evaluation of response rate and survival was performed in only breast cancer. Histological criteria made by Japanese Breast Cancer Society for assessment of therapeutic response was used to evaluate pathological response to NAC [21]. As shown in Figure 4A, there was no significant correlation between HMGB1 score in pre-treatment samples and pathological response after chemotherapy, and between HMGB1 score in after-treatment samples and the pathological response. Overall survival in responder group to chemotherapy was significantly superior to that in non-responder group (p = 0.0394, Figure 4B). However, there were no significant difference in the survival data between HMGB1-high score and -low score in the pre-treatment samples (p = 0.9130, Figure 4B). Similarly, calreticulin expression in the pre-treatment samples did not affect the pathological response nor the overall survival (Figure 5A and 5B).

Taken together, HMGB1 or calreticulin score in the pre-treatment samples is not a useful biomarker to predict pathological response to chemotherapy.

***In vitro* treatment of breast cancer cell lines with anticancer drugs**

To further evaluate HMGB1 and calreticulin expression following chemotherapy, 3 breast tumor cell lines were treated with paclitaxel or doxorubicin *in vitro* and, the production of HMGB1 and surface expression of calreticulin, along with the proportion of dying cells (Figure 6A), were analyzed.

As shown in Figure 6B and 6C, chemotherapeutic drugs alone could induce variable levels of HMGB1 production (Figure 6B) and surface calreticulin

expression (Figure 6C) depending on the drug and among cell line, regardless of almost the same amount of dying cells.

Discussion

The present study contains novel findings to support the concept of immunogenic cell death induced by chemotherapy alone in patients with breast cancer and esophageal cancer. First, both HMGB1 and calreticulin expression were significantly upregulated after chemotherapy. Second, chemotherapeutic drugs induced upregulation of HMGB1 and calreticulin in several breast cancer cell lines tested.

We and others have been recently shown that danger signals from dying cells treated by radiotherapy or some chemotherapeutic drugs, such as anthracyclines and oxaliplatin, could induce TLR-dependent, antigen-specific T-cell immunity [22, 23]. Additional therapeutic modalities that have been shown to induce ICD include oncolytic virus therapy [24-26] and photodynamic therapy [27, 28]. Furthermore, among various danger signals released from dying cells in the tumor-bearing mouse model, HMGB1, but not other known TLR4 ligands, could be a mandatory factor to induce tumor antigen–specific T-cell immunity [22, 23]. In the present study, we showed for the first time in a human clinical study that conventional chemotherapy alone significantly induced up-regulation of HMGB1 in breast and esophageal cancer. Unfortunately, there was no correlation between degree of HMGB1 and pathological response after chemotherapy or between degree of HMGB1 and patient`s survival.

Moreover, it has been shown that early membrane exposure of calreticulin induced by some chemotherapeutic agents such as anthracyclines and oxaliplatin [15, 23, 29-31] could enhance phagocytosis of dying tumor cells by DCs in vitro [32-34], and both HMGB1 release and calreticulin cell surface

expressions are required for antigen-specific T-cell response in murine model. However, we could not show any significant differences in pathological response level and overall survival between calreticulin high and low in pre- and post-chemotherapy samples. Further study will be needed to address the more detailed mechanisms behind which mediators are associated with ICD in response to chemotherapy.

Of interest, we showed that conventional chemotherapy alone could significantly induce upregulation of HMGB1 and calreticulin in patients with breast or esophageal cancer. Also, the *in vitro* study indicated that there were substantial variations in HMGB1 production following chemotherapy depending on breast cancer cell lines regardless of almost the same amount of dying cells. These observations suggest that immune reactions related to ICD following chemotherapy may affect clinical outcomes in patients with breast cancer. In the present study, expression of HMGB1 or calreticulin is not able to predict pathological response or patient`s survival. However, Apetoh and colleagues reported that patients with breast cancer with a TLR4 loss-of-function allele relapse more quickly after chemotherapy than those with a normal TLR4 allele [23], indicating a clinically relevant immune reaction triggered by TLR-dependent ICD induced by chemotherapy. In clinical setting with patient of esophageal cancer, we have reported that HMGB1 production is related to clinical outcome after chemoradiation [35]. Thus, although there is still controversy among tumor types or therapeutic modality, it is likely that ICD-related immune response after chemotherapy may play an important role in clinical outcome of cancer treatments.

In conclusion, the present study strongly indicated that chemotherapy alone can induce ICD.

Figure Legends

Figure 1. Representative immunostainings for HMGB1 and calreticulin in breast (A) and esophageal cancer (B) using anti-HMGB1, anti-calreticulin mAbs. The grade of HMGB1 and calreticulin expression was scored as 0 (0%–10% positive), 1+ (10%–30% positive), 2+ (30%–80% positive), or 3+ (> 80% positive) based on the tumor cells.

Figure 2. Representative immunostainings of HMGB1 and calreticulin before and after neoadjuvant chemotherapy in breast (A) and esophageal cancer (B).

Figure 3. Summarized data for semi-quantitative evaluation of HMGB1 and calreticulin expression before and after neoadjuvant chemotherapy in breast and esophageal cancer.

Figure 4. HMGB1 expression relating to clinical outcomes.
(A) HMGB1 expression relating to pathological response was shown in breast cancer patients. Chi-square test was used for the evaluation between chemo-response and HMGB1 expression. Histological criteria made by Japanese Breast Cancer Society for assessment of therapeutic response was used to evaluate pathological response to NAC. (B) Pathological response relating to overall survival (left graph) and HMGB1 expression before neoadjuvant chemotherapy relating to overall survival (right graph).

Figure 5. Calreticulin expression relating to clinical outcomes.
(A) Calreticulin expression relating to pathological response in breast cancer

patients. (B) Calreticulin expression before neoadjuvant chemotherapy relating to overall survival.

Figure 6. *In vitro* treatment of breast cancer cell lines with chemotherapeutic drugs. (A) The proportion of dying cells [Annexin-V (+) or 7-AAD (+)] was analyzed by flow cytometry. (B) HMGB1 in the supernatant after chemotherapy were evaluated by ELISA. (C) Calreticulin expressed on cell surface after chemotherapy were evaluated by flow cytometry.
PTX, paclitaxel; DXR, doxorubicin;

References

1. Ishikura, S., et al., *Long-Term Toxicity After Definitive Chemoradiotherapy for Squamous Cell Carcinoma of the Thoracic Esophagus.* 2003.
2. Manzoni, G.d., et al., *Induction Chemoradiotherapy for Squamous Cell Carcinoma of the Thoracic Esophagus: Impact of Increased Dosage on Long-Term Results.* The Annals of Thoracic Surgery, 2005. **80**(4): p. 1176-1183.
3. *Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials.* The Lancet, 2005. **365**(9472): p. 1687-1717.
4. Wolmark, N., et al., *Preoperative chemotherapy in patients with operable breast cancer: nine-year results from National Surgical Adjuvant Breast and Bowel Project B-18.* J Natl Cancer Inst Monogr, 2001(30): p. 96-102.
5. Bear, H.D., et al., *The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27.* J Clin Oncol, 2003. **21**(22): p. 4165-74.
6. Rastogi, P., et al., *Preoperative chemotherapy: updates of National Surgical Adjuvant Breast and Bowel Project Protocols B-18 and B-27.* J Clin Oncol, 2008. **26**(5): p. 778-85.
7. Kono, K. and K. Mimura, *Immunogenic tumor cell death induced by chemoradiotherapy in a clinical setting.* Oncoimmunology, 2013. **2**(1): p. e22197.
8. Kono, K., K. Mimura, and R. Kiessling, *Immunogenic tumor cell death*

induced by chemoradiotherapy: molecular mechanisms and a clinical translation. Cell Death Dis, 2013. 4: p. e688.

9. Kroemer, G., et al., *Immunogenic cell death in cancer therapy.* Annu Rev Immunol, 2013. **31**: p. 51-72.
10. Krysko, D.V., et al., *Immunogenic cell death and DAMPs in cancer therapy.* Nat Rev Cancer, 2012. **12**(12): p. 860-75.
11. Krysko, O., et al., *Many faces of DAMPs in cancer therapy.* Cell Death Dis, 2013. 4: p. e631.
12. Ladoire, S., et al., *Immunogenic cell death-related biomarkers: Impact on the survival of breast cancer patients after adjuvant chemotherapy.* Oncoimmunology, 2016. **5**(2): p. e1082706.
13. Stoll, G., et al., *Immune-related gene signatures predict the outcome of neoadjuvant chemotherapy.* Oncoimmunology, 2014. **3**(1): p. e27884.
14. Gebremeskel, S. and B. Johnston, *Concepts and mechanisms underlying chemotherapy induced immunogenic cell death: impact on clinical studies and considerations for combined therapies.* Oncotarget, 2015. **6**(39): p. 41600-19.
15. Casares, N., et al., *Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death.* J Exp Med, 2005. **202**(12): p. 1691-701.
16. Wong, D.Y., W.W. Ong, and W.H. Ang, *Induction of immunogenic cell death by chemotherapeutic platinum complexes.* Angew Chem Int Ed Engl, 2015. **54**(22): p. 6483-7.
17. Galluzzi, L., et al., *Immunological Effects of Conventional Chemotherapy and Targeted Anticancer Agents.* Cancer Cell, 2015. **28**(6): p. 690-714.
18. Hodge, J.W., et al., *Chemotherapy-induced immunogenic modulation of tumor cells enhances killing by cytotoxic T lymphocytes and is distinct from immunogenic cell death.* Int J Cancer, 2013. **133**(3): p. 624-36.
19. Kepp, O., et al., *Consensus guidelines for the detection of immunogenic cell death.* Oncoimmunology, 2014. **3**(9): p. e955691.

20. Okano, M., et al., *Upregulated Annexin A1 promotes cellular invasion in triple-negative breast cancer.* Oncol Rep, 2015. **33**(3): p. 1064-70.
21. Masafumi, K., et al., *Histological criteria for assessment of therapeutic response in breast cancer (2007 version).* Breast Cancer, 2007. **15**: p. 5–7.
22. Apetoh, L., et al., *The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy.* Immunological Reviews, 2007. **220**(1): p. 47-59.
23. Apetoh, L., et al., *Toll-like receptor 4 | [ndash] | dependent contribution of the immune system to anticancer chemotherapy and radiotherapy.* Nature Medicine, 2007. **13**(9): p. 1050-1059.
24. Miyamoto, S., et al., *Coxsackievirus B3 is an oncolytic virus with immunostimulatory properties that is active against lung adenocarcinoma.* Cancer Res, 2012. **72**(10): p. 2609-21.
25. Diaconu, I., et al., *Immune response is an important aspect of the antitumor effect produced by a CD40L-encoding oncolytic adenovirus.* Cancer Res, 2012. **72**(9): p. 2327-38.
26. Takasu, A., et al., *Immunogenic cell death by oncolytic herpes simplex virus type 1 in squamous cell carcinoma cells.* Cancer Gene Ther, 2016. **23**(4): p. 107-13.
27. Garg, A.D., et al., *A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death.* Embo j, 2012. **31**(5): p. 1062-79.
28. Tanaka, M., et al., *Immunogenic cell death due to a new photodynamic therapy (PDT) with glycoconjugated chlorin (G-chlorin).* Oncotarget, 2016.
29. Michaud, M., et al., *Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice.* Science, 2011. **334**(6062): p. 1573-7.
30. Zappasodi, R., et al., *Improved clinical outcome in indolent B-cell lymphoma patients vaccinated with autologous tumor cells experiencing immunogenic death.* Cancer Res, 2010. **70**(22): p. 9062-72.

31. Fucikova, J., et al., *Human tumor cells killed by anthracyclines induce a tumor-specific immune response.* Cancer Res, 2011. **71**(14): p. 4821-33.
32. Tesniere, A., et al., *Immunogenic death of colon cancer cells treated with oxaliplatin.* Oncogene, 2009. **29**(4): p. 482-491.
33. Zitvogel, L., et al., *Immunogenic tumor cell death for optimal anticancer therapy: the calreticulin exposure pathway.* Clin Cancer Res, 2010. **16**(12): p. 3100-4.
34. Obeid, M., et al., *Calreticulin exposure dictates the immunogenicity of cancer cell death.* Nat Med, 2007. **13**(1): p. 54-61.
35. Suzuki, Y., et al., *Immunogenic tumor cell death induced by chemoradiotherapy in patients with esophageal squamous cell carcinoma.* Cancer Res, 2012. **72**(16): p. 3967-76.

Table 1. Patient characteristics

Breast Cancer	n=52
Age	
Range	26–75
Median	53
T1	7
T2	24
T3	7
T4	14
N0	2
N1	41
N2	7
N3	2
M0	50
M1	2
Stage	
Ⅰ	0
Ⅱ	27
Ⅲ	23
Ⅳ	2
Subtype	
luminal	21
HER2	5
Triple negative	17
luminal + HER2	8

UICC TNM classification

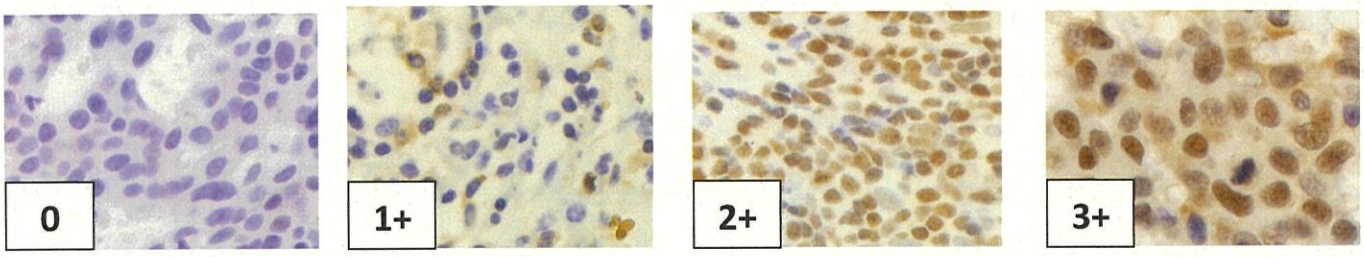
Esophageal Cancer	n=8
Age	
Range	22–77
Median	58
T1	1
T2	3
T3	2
T4	1
N0	1
N1	3
N2	3
N3	1
M0	7
M1	1
Stage	
Ⅰ	0
Ⅱ	2
Ⅲ	5
Ⅳ	1

UICC TNM classification

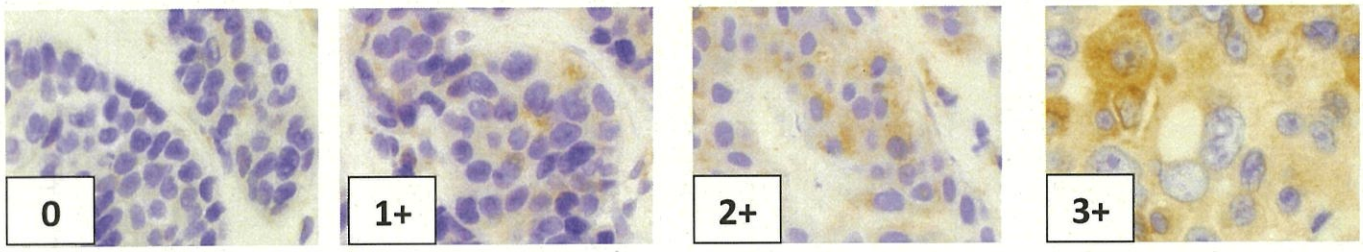
A

Breast cancer

HMGB1



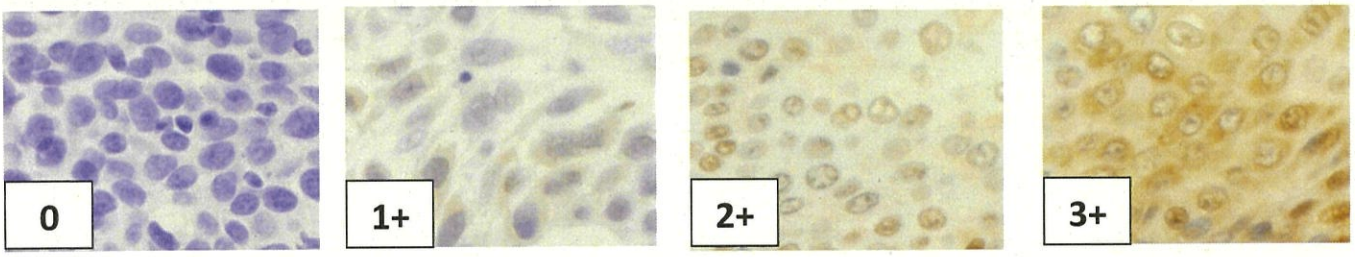
Calreticulin



B

Esophageal cancer

HMGB1



Calreticulin

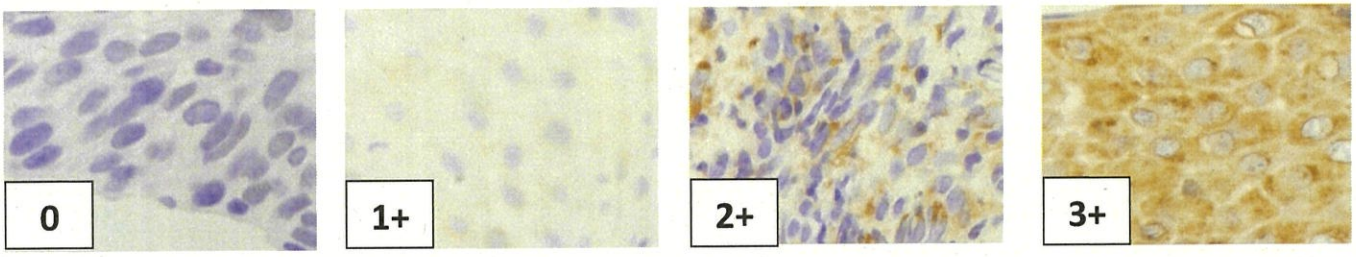
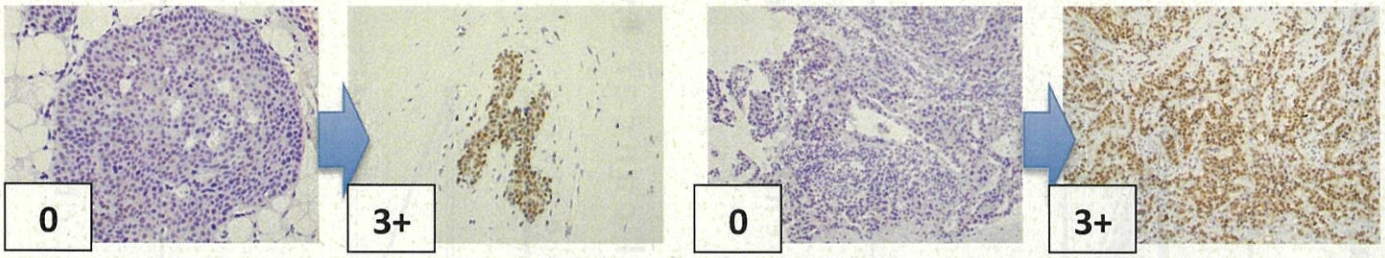


Figure 1

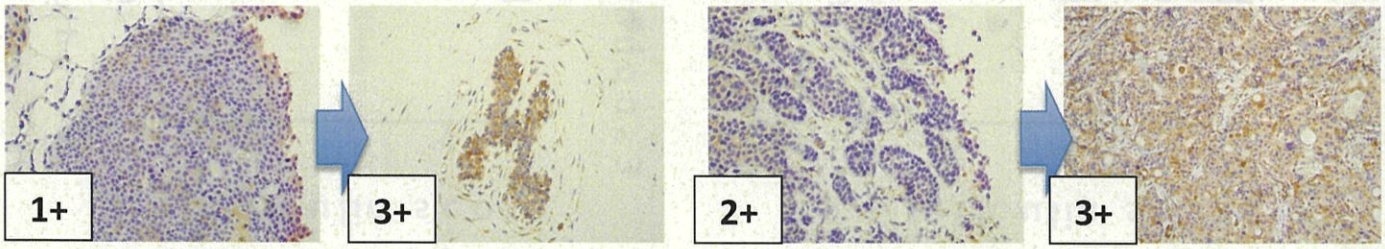
A

Breast cancer

HMGB1



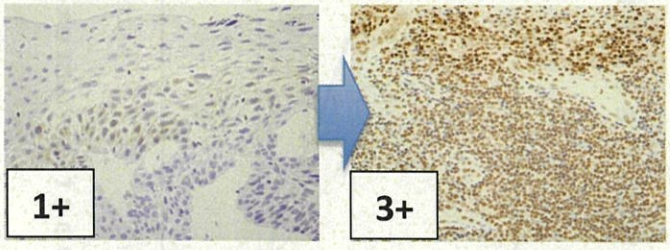
Calreticulin



B

Esophageal cancer

HMGB1



Calreticulin

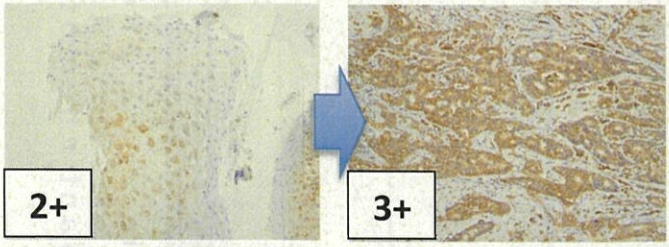
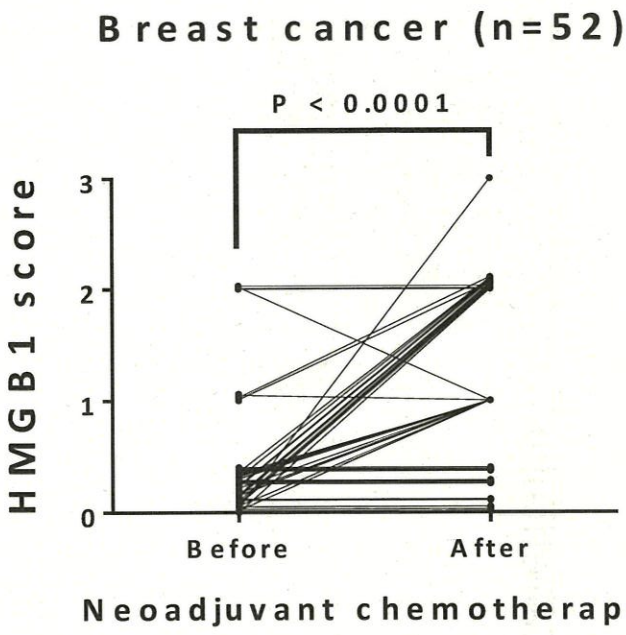
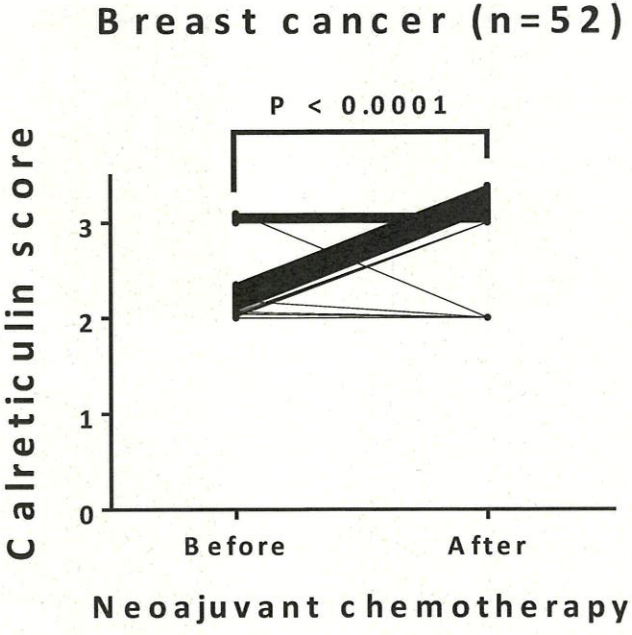
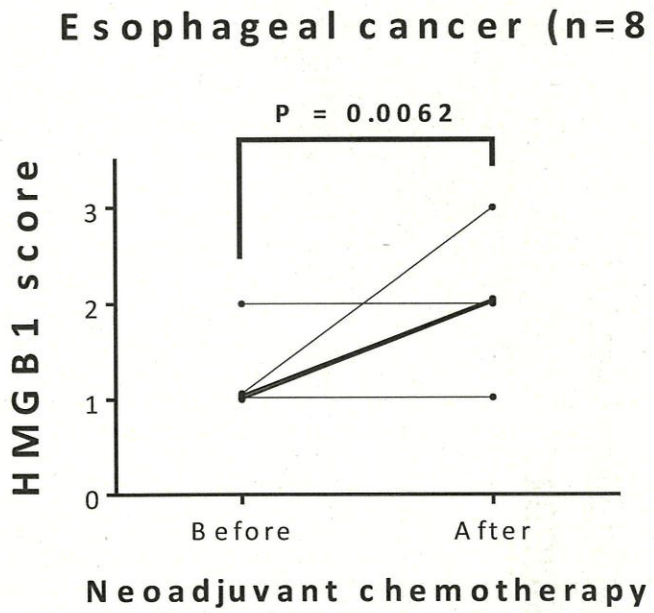


Figure 2


Breast cancer (n=52)
P < 0.0001
HMGB1 score
3
2
1
0
Before
After
Neoadjuvant chemotherapy


Breast cancer (n=52)
P < 0.0001
Calreticulin score
3
2
1
0
Before
After
Neoajuvant chemotherapy


Esophageal cancer (n=8)
P = 0.0062
HMGB1 score
3
2
1
0
Before
After
Neoadjuvant chemotherapy

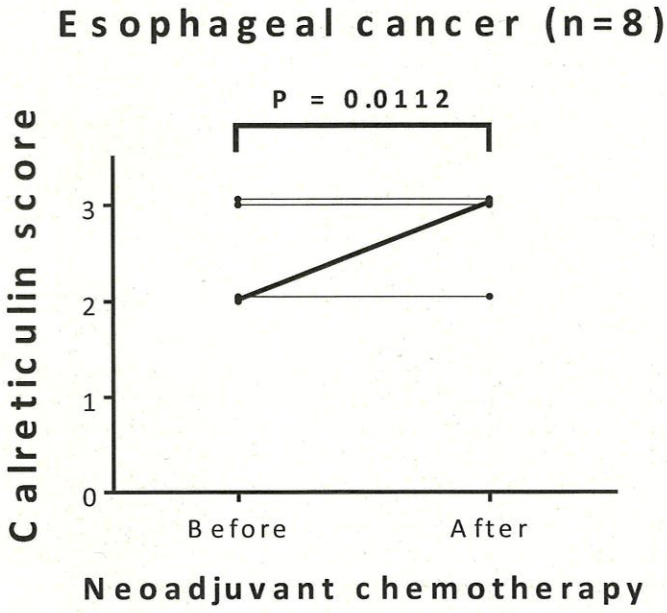

Esophageal cancer (n=8)
P = 0.0112
Calreticulin score
3
2
1
0
Before
After
Neoadjuvant chemotherapy

Figure 3

A

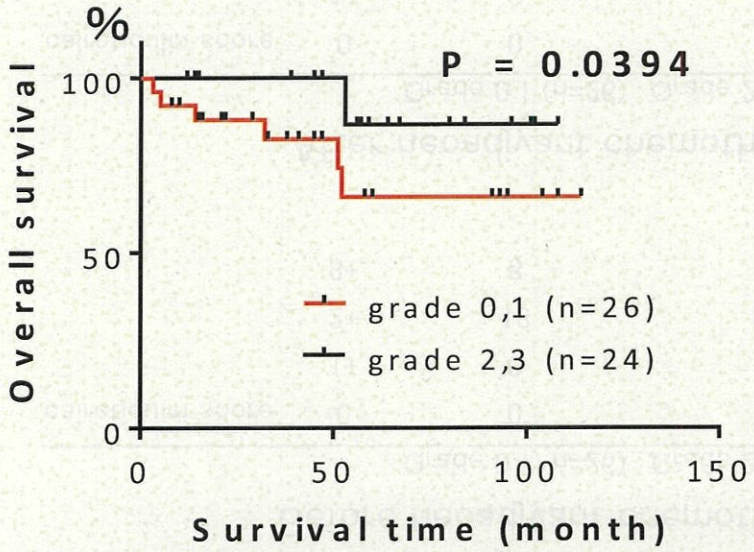
Before neoadjvant chemotherapy

		Grade 0,1 (n=26)	Grade 2,3 (n=24)	Total	P Value
HMGB1 score	0	23	18	41	0.4648
	1+	2	4	6	
	2+	1	2	3	
	3+	0	0	0	

After neoadjvant chemotherapy

		Grade 0,1 (n=26)	Grade 2,3 (n=24)	Total	P Value
HMGB1 score	0	5	8	13	0.5076
	1+	5	6	11	
	2+	8	6	14	
	3+	8	4	12	

B



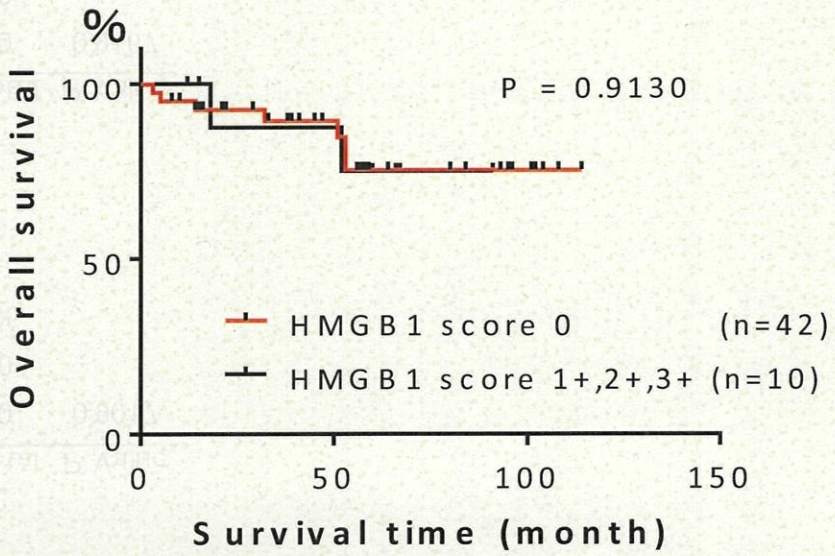


Figure 4

A

Before neoadjvant chemotherapy

		Grade 0,1 (n=26)	Grade 2,3 (n=24)	Total	P Value
calreticulin score	0	0	0	0	0.9017
	1+	0	0	0	
	2+	18	17	35	
	3+	8	7	15	

After neoadjvant chemotherapy

		Grade 0,1 (n=26)	Grade 2,3 (n=24)	Total	P Value
calreticulin score	0	0	0	0	0.9167
	1+	0	0	0	
	2+	3	3	6	
	3+	23	21	44	

B

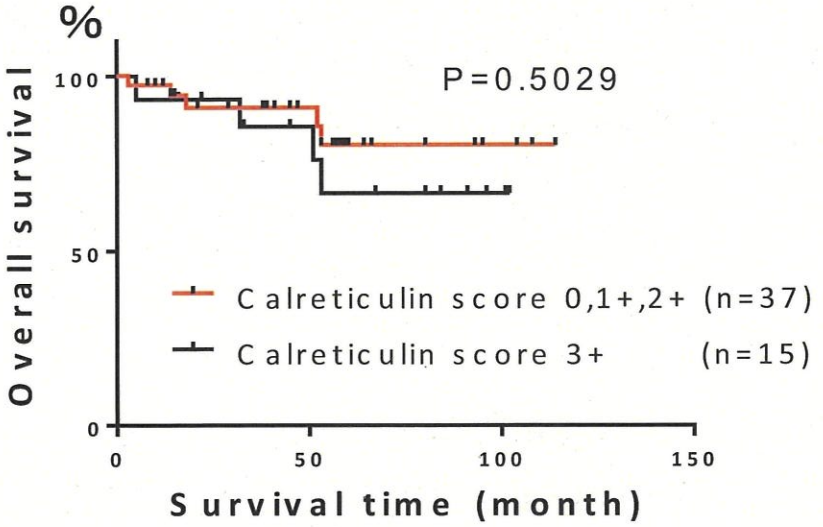


Figure 5

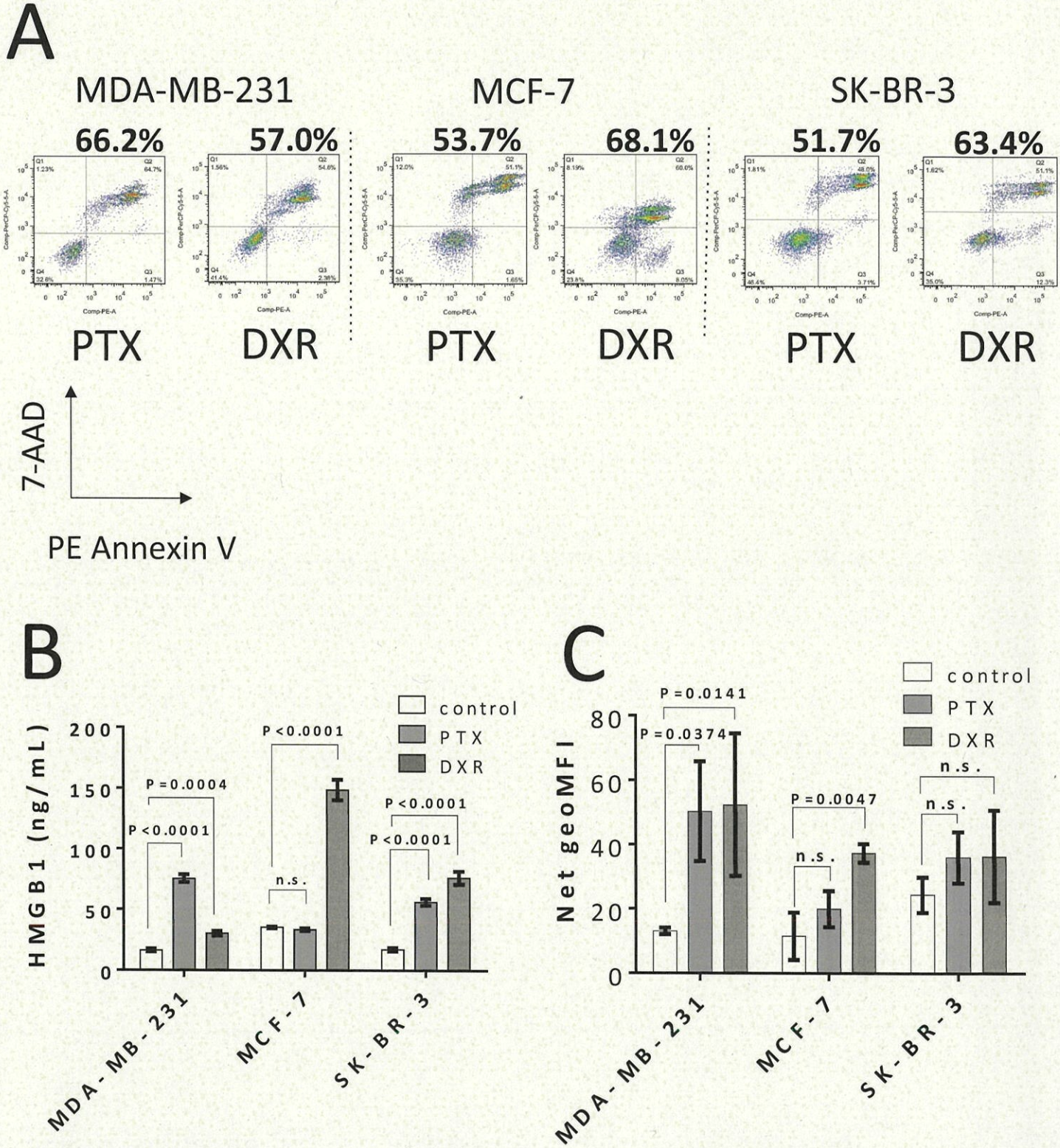

A
MDA-MB-231
MCF-7
SK-BR-3
66.2%
57.0%
53.7%
68.1%
51.7%
63.4%
PTX
DXR
PTX
DXR
PTX
DXR
7-AAD
PE Annexin V
B
HMGB1 (ng/mL)
control
PTX
DXR
P = 0.0004
P < 0.0001
P < 0.0001
n.s.
P < 0.0001
P < 0.0001
MDA-MB-231
MCF-7
SK-BR-3
C
Net geoMFI
control
PTX
DXR
P = 0.0141
P = 0.0374
P = 0.0047
n.s.
n.s.
n.s.
MDA-MB-231
MCF-7
SK-BR-3
MDA-MB-231
MCF-7
SK-BR-3
Count
PE-A
Negative control
No treatment control
PTX
DXR

Figure 6